

AD_____

Award Number: DAMD17-97-1-7093

TITLE: Mevalonates, Ras and Breast Cancer

PRINCIPAL INVESTIGATOR: Michael A. White, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas Southwestern
Medical Center at Dallas
Dallas, Texas 75235

REPORT DATE: September 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20020904 014

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 074-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503				
1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE September 2001		3. REPORT TYPE AND DATES COVERED Final (1 Sep 97 - 31 Aug 01)
4. TITLE AND SUBTITLE Mevalonates, Ras and Breast Cancer			5. FUNDING NUMBERS DAMD17-97-1-7093	
6. AUTHOR(S) Michael A. White, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The University of Texas Southwestern Medical Center at Dallas Dallas, Texas 75235 E-Mail: michael.white@utsouthwestern.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES Report contains color				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) Therapy of any cancer is designed to control growth of cancer cells while permitting the function and proliferation of normal cells. 6-fluoromevalonate (Fmev) can inhibit proliferation of leukemic cells lines while not affecting normal lymphocytes. This selective inhibition appears to be a consequence of expression of oncogenic Ras. Here we are evaluating the ability of Fmev to selectively interfere with proliferation of breast cancer cells. We have shown that Fmev can inhibit the proliferation of normal breast epithelial cells. However the proliferation of, some, but not all, transformed breast epithelial cell lines expressing oncogenic Ras is inhibited by concentrations of Fmev that do not affect normal breast epithelia. Our study of the contribution of oncogenic Ras to this sensitization, suggests that it is an indirect mechanism involving reduced adherence to substrate. In addition, while growth of ras transformed cells on plastic is inhibited by Fmev, growth in suspension is not. Our results do not support pursuing Fmev as an effective therapy against Ras transformed breast epithelia.				
14. SUBJECT TERMS Breast Cancer			15. NUMBER OF PAGES 11	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

Table of Contents

Cover	1
SF 298	2
Table of Contents	3
Introduction	4
Body	4
Key Research Accomplishments	10
Reportable Outcomes	10
Conclusions	10
References	11
Appendices	NA

Introduction

The subject of this research project is potential breast cancer therapy through the use of agents that inhibit the proliferation of malignant cells while allowing the proliferation and function of normal cells. The purpose of this work is to: 1) evaluate the efficacy of a mevalonate analogue, 6-fluoromevalonate (Fmev), in selective inhibition of breast cancer cell growth, and 2) to determine the molecular mechanism by which Fmev can inhibit proliferation of transformed cells. The scope of this research is confined to analysis of the effects of Fmev on proliferation and mitogenic signal transduction in breast epithelial cells, and breast tumor-derived cell lines with a focus on the consequences of oncogenic Ras expression.

Body

Award DAMD17-97-1-7093 originally to Dr. J. Cuthbert was functionally transferred to my laboratory on or about December 1, 1999. This final report covers our progress from December 1, 1999 to August 31, 2001.

Original Statement of Work Task 1: Intracellular mevalonates regulate Ras in breast cancer cells.

The fluorinated mevalonate analog, 6-fluoromevelonate (Fmev) blocks the conversion of mevalonate diphosphate to isopentenyl diphosphate. This is a critical step in the metabolism of acetate to farnesyl diphosphate. Farnesyl diphosphate is used for the post-translation modification of some proteins, such as Ras, by farnesylation or

geranylgeranylation. Inhibition of this post-translational modification can block the function of some Ras isoforms in cells. Dr. Cuthbert has shown that treatment of leukemia- and lymphoma-derived cell lines with Fmev depletes the cellular pool of Ras protein. In addition, Dr. Cuthbert has demonstrated that Fmev selectively inhibits growth of leukemic cell lines as compared to normal lymphocytes (3,4). This task was directed at examining the effects of Fmev on Ras levels in breast cancer cells. Analysis was to include regulation at the level of gene transcription, as well as at the level of protein stability. This task had not yet been attempted when the project was transferred to my laboratory (beginning of year 3 of the project).

Fmev is not a commercially available compound. Therefore, our initial effort was to synthesize a supply of Fmev in collaboration with Dr. C. Falck (UT Southwestern). The synthesis reactions were carried out according to the methods of Quistad (1).

Our next effort was to evaluate the sensitivity of normal diploid breast epithelial cells to the anti-proliferative effects of Fmev. We chose HME50-5E as our model cell line as it mimics the normal growth characteristics of primary cell lines in culture but will not undergo replicative senescence due to a spontaneous immortalization event (2). As shown in Figure 1, the proliferation of HME50-hTERT is affected by Fmev with an approximate IC₅₀ of 20 μ M final concentration. Consistent with Dr. Cuthbert's reported results in leukemic cell lines (3), the total levels of cellular Ras were reduced in HME50-hTERT cells treated with concentrations of Fmev that inhibited proliferation. Levels of active ERK1 and ERK2, MAP kinases regulated by Ras, were also reduced by Fmev. As

activation of ERK1/2 directly correlates with cell cycle progression, these results suggest a possible mechanistic explanation for the growth-inhibitory effects of Fmev.

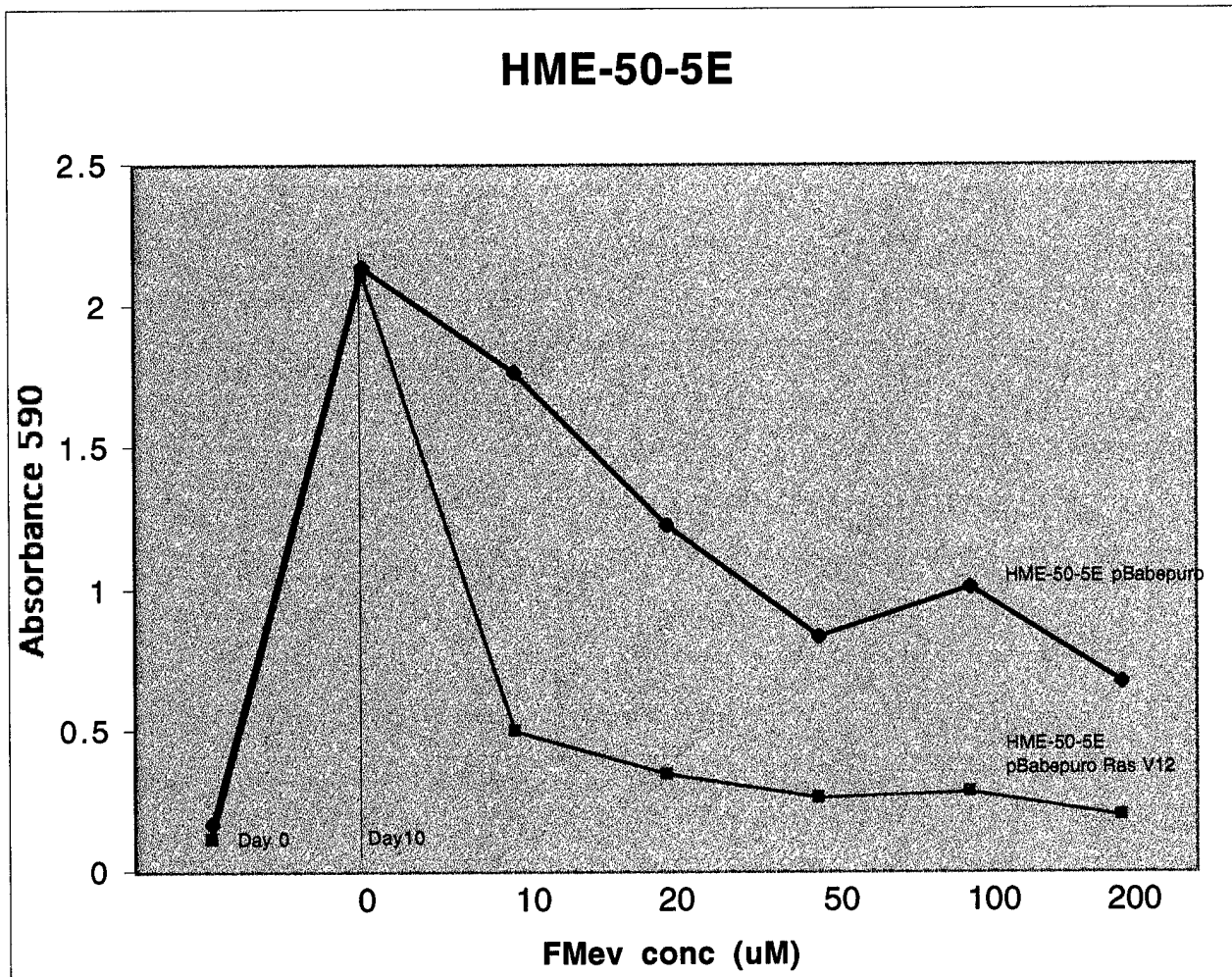


Figure 1. HME50-5E and HME50-5E-ras12V cells were plated at a density of 10,000 cells/well in 24 well plates with the indicated concentrations of Fmev. Following 10 days of culture, cells were fixed and stained with Giemsa. After extensive washing, the stain was extracted with acetic acid, and A590 was measured. The A590 of the starting density is shown for comparison (day 0).

Dr. Cuthbert's earlier work suggests that oncogenic Ras expressing cells may have increased sensitivity to Fmev (4), and that transfection of lymphocytes with oncogenic Ras resulted in increased sensitivity to the growth-inhibitory effects of Fmev. To test this directly, we established a line of HME-50-5E that stably express H-ras12V from a retroviral LTR promoter. These cells have elevated levels of active ERK1/2 under serum-deprived growth conditions as compared to the parental cells. As shown in Figure 1, ras12V expressing cells are markedly more sensitive to the growth inhibitory effects of Fmev. In contrast to parental cells, the levels of active ERK in ras12V expressing are not detectably affected by Fmev. In addition, we did not detect any reduction in levels of Ras expression. These observations suggest that the growth inhibitory effects of Fmev on cells sensitized by ras12V are not due to inhibition of the mitogenic signaling of Ras to ERK. Other Ras-dependent pathways may be involved. The sensitization of cells to Fmev by Ras12V may be downstream of ERK regulation, or through other Ras effector pathways.

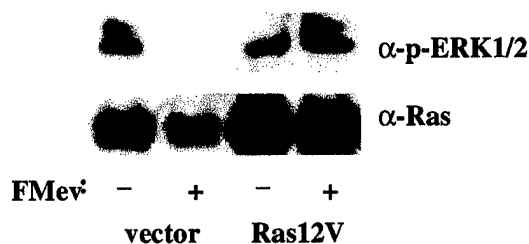


Figure 2. HME50-5E were infected with empty pBabePuro (vector) or with pBabePuro-ras12V (Ras12V). Whole cell lysates were prepared from cells treated or not with 100 uM Fmev for 48 hours. Lysates were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

Original Statement of Work, Task 2: Mechanism(s) of Fmev- and lovastatin-induced changes in Ras levels.

Given our observations that transfection of oncogenic Ras was sufficient to sensitize cells to the growth inhibitory effects of Fmev, and that Fmev had no effect on the expression

of oncogenic Ras (which in this case was Hras), we concluded that if the inhibitory effects of Fmev were a consequence of reduced cellular Ras levels (as stated in the hypothesis of the original proposal) it must be a consequence of reduction of specific Ras isoform. However, analysis of levels of Kras and Nras in Fmev-treated HME50-5E-ras12V cells revealed no differences as compared to untreated cells. Therefore, this task was flawed in that our analysis suggested that inhibition of growth by Fmev is independent of changes in levels of total Ras protein. This does not rule out the possibility that the effects of Fmev are through altered compartmentalization of Ras proteins. We did not have time or funds to pursue this latter possibility.

Original Statement of Work, Task 3. Functional effects of Fmev- and lovastatin inhibitors on apoptosis and cell proliferation.

We observed an increased sensitivity to the growth inhibitory effects of Fmev on HME-ras12V cells as compared to HME cells. These assays were performed using normal tissue-culture growth conditions for adherent cells. Given this, possible mechanisms for the reduced growth rates observed include induction of apoptosis, cell cycle arrest, or reduced adherence of cells to substrate. The latter possibility would, of course, be the least interesting in the context of evaluating potential therapeutic applications of Fmev to tumor cell growth. Therefore, we next evaluated the consequences of Fmev exposure to growth of HME-ras12V cells in soft agar.

HME50-hTERT expressing cells like normal human epithelial cells, display anchorage-dependent growth. Incubation of cells in soft-agar cultures blocks proliferation and results in apoptosis. We and others (5) have found that normal human epithelial cells can

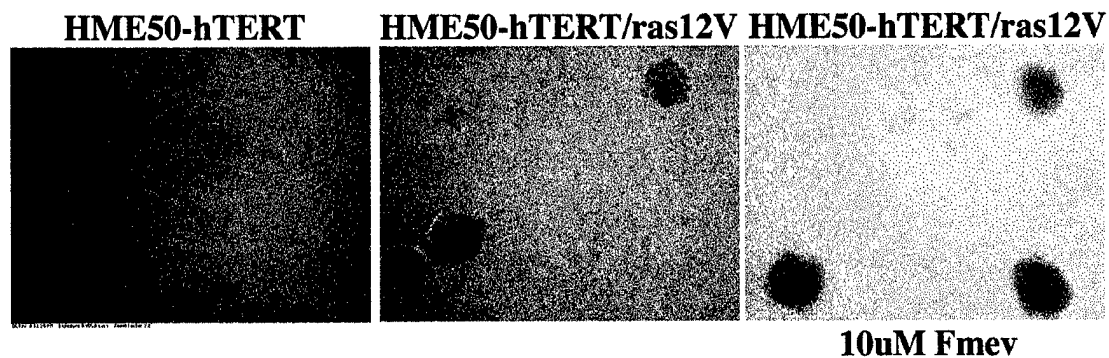


Figure 3. Fmev does not inhibit growth of Ras-transformed breast epithelial cells in soft agar. For each indicated cell line, 5000 cells were plated in 0.3% agar and incubated in defined media with or without Fmev at the indicated concentration. Colonies were photographed following 14 days of incubation. Representative images are shown.

be progressed to a transformed state by introduction of telomerase, oncogenic Ras, and (in the case of the Weinberg group) large and small T-antigen. In our hands, introduction of T-antigen is unnecessary as long as the cells are mechanically released from culture dishes prior to incubation in soft agar, rather than by trypsin. This gives us a model system to study transformation of human mammary epithelial cells in response to a defined series of changes, none of which involves introduction of viral oncogenes. Using this model system, we assessed the ability of Fmev to inhibit soft-agar colony formation by HME-hTERT-ras12V cells. As shown in figure 3, even doses two-fold above those that will completely inhibit growth on plastic, has no effect on colony formation in soft agar. One likely explanation for this result, is that our original observations of the inhibitory effects of Fmev were due to drug induced loss of anchorage. As a

consequence of these results, and as a consequence of the end of the project period, we have not further pursued these studies.

Key Research Accomplishments

1. Synthesis of Fmev
2. Characterization of the sensitivity of human mammary epithelial cells to growth-inhibitory effects of Fmev.
3. Characterization of the sensitivity of oncogenic Ras expressing human mammary epithelial cells to growth-inhibitory effects of Fmev.
4. Characterization of the effects of Fmev on Ras protein levels.
5. Development of a defined model system to study growth transformation of human mammary epithelial cells (HME-hTERT/Ras12V).
6. Characterization of the effects of Fmev on the anchorage-independent growth of transformed HME cells.

Reportable Outcomes: none to date.

Conclusions

We have established that, at appropriate concentrations, Fmev can preferentially inhibit the growth of human mammary epithelial cells expressing oncogenic Ras versus those that do not. These results give an initial indication that Fmev may have utility as an antagonist to breast cancer cell growth. Further studies demonstrated that, contrary to the original hypothesis, Fmev did not affect levels of Ras protein. In addition, Fmev did not inhibit anchorage independent growth of transformed cells. We conclude that Fmev is

not a promising therapeutic approach as defined by the hypothesis laid out in the original proposal.

References

1. Quistad, G.B, Cerf, D. C., Schooley, D. A., and Staal, G. B. (1981). Fluoromevalonate-an inhibitor of insect juvenile hormone biosynthesis. *Pr Nauk Inst Chem Org Fiz Politech Wroclaw* **22**: 163-168.
2. Shay, J. W., Tomlinson, G., Piatyszek, M. A., and Gollahon, L. S. (1995). Spontaneous in vitro immortalization of breast epithelial cells from a patient with Li-Fraumeni syndrome. *Mol. Cell. Biol.* **15**: 425-432.
3. Cuthbert, J. A., and Lipsky, P. E. (1995). Suppression of the proliferation of Ras-transformed cells by fluoromevalonate, an inhibitor of mevalonate metabolism. *Cancer Res.* **55**: 1732-1740.
4. Cuthbert, J. A., and Lipsky, P. E. (1997). Regulation of proliferation and Ras localization in transformed cells by products of mevalonate metabolism. *Cancer Res* **57**: 3498-3505.
5. Elenbaas B, Spirio L, Koerner F, Fleming MD, Zimonjic DB, Donaher JL, Popescu NC, Hahn WC, Weinberg RA. (2001). Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. *Genes Dev.* **15**: 50-65.